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Gene silencing with STAT6 specific siRNAs blocks eotaxin release in IL-4/TNFα stimulated human epithelial cells

Jörg F. Rippmann, Andreas Schnapp, Andreas Weith, Silke Hobbie*

Department of Pulmonary Research, Boehringer Ingelheim Pharma GmbH & Co. KG, Birkendorfer Straße 65, 88397 Biberach an der Riss, Germany

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Abstract **Small interfering RNAs have evolved as effective tools for the study of gene functions. Here, we demonstrate the use of different siRNAs for the specific knock down of the STAT6 transcription regulator and the complete silencing of the downstream signaling pathway. The knock down of STAT6 resulted in a complete loss of STAT6 specific DNA binding activity and blocked the release of eotaxin-3 in human epithelial cells (BEAS-2B) stimulated with IL-4 and TNFα with no signs of unspecific gene silencing. Other signaling pathways like the EGF stimulated release of IL-8 were still active in BEAS-2B cells treated with STAT6 specific siRNAs, demonstrating the specificity of these molecules.**

Keywords: RNA interference; siRNA; STAT6; IL-4; Eotaxin; Asthma

1. Introduction

Asthma, an increasingly common disease characterized by airway obstruction and bronchial hyper-responsiveness (BHR), is caused by acute and chronic bronchial inflammation. The molecular and cellular mechanisms mediating the allergic inflammatory cascade involve multiple mediators, cell types and pathways. T helper type 2 (Th2) cytokines like IL-4 and IL-13 are believed to play a central role in the initiation and the progression of this disease [1]. For example, IL-4 is essential for the commitment of naïve CD4+ T-cells into the Th2-phenotype and Th2 cell proliferation, whereas IL-13 is known to affect BHR. In addition, IL-4 and IL-13 share several biological responses due to a common signaling pathway. Both cytokines promote eosinophilic inflammation through induction of eotaxin production in bronchial epithelial cells and fibroblasts [2]. IL-4 and IL-13 also increase airway mucus secretion and both are required for the induction of the IgE isotype switch.

The key event for IL-4 and IL-13 mediated biological effects is the activation of the signal transducer and activator of transcription 6 (STAT6) [3]. STAT6 belongs to a family of transcription regulators that mediate signal transduction upon binding to phosphorylated receptors via a SH2 domain.

Phosphorylation by receptor bound JAK-kinases is then followed by a SH2 domain dependent homo- or hetero-dimer formation in the cytoplasm. Subsequent translocation into the nucleus causes recognition of STAT6 specific DNA-binding elements and enhanced transcription of target genes. The JAK/STAT signaling pathways are stringently controlled and a negative feedback control is the competition of the silencers of cytokine signaling (SOCS) with STATs for the JAK-kinase activation mechanism [4]. Especially for the STAT6 signaling, a silencing through the IFNγ/STAT1 pathway via the induction of SOCS1 gene expression was demonstrated in different cell lines [5–7].

The main effects triggered by IL-4 and IL-13 are reduced or eliminated in allergen provocation experiments in STAT6−/− mice [8], implying that the inhibition of STAT6-mediated transcription might reduce the IL-4 and IL-13 associated features of human asthma.

Small molecule drugs able to interfere with the functional domains of STAT6 (e.g., the DNA-binding domain or the SH2 domain) have not been identified so far. Therefore "biological therapies" emerge as potential alternatives to target this important pathway. One of the most promising technologies takes advantage of the intracellular recognition of double stranded RNA for the posttranscriptional silencing of gene expression [9]. This RNA interference (RNAi) was first described in plants but the principal mechanism has also been demonstrated in worms, flies and mammalian cells [10,11]. Double stranded RNA is cleaved by Dicer, a RNAse III nuclease family member, and processed into 21–28 nucleotide small interfering RNAs (siRNAs) [12]. These double stranded oligonucleotides are incorporated into a RNA-induced silencing complex (RISC), which cleaves the target mRNA sequence. Specificity of the silencing is very high and even single mismatches are weakly tolerated [13]. However, in mammalian cells, unspecific effects have been described once the siRNA concentration is high enough to activate a general silencing of gene expression by the interferon pathway, indicated by a five to tenfold upregulation of STAT1 [14]. In addition, specific binding of the siRNA to homologous sequences [15] or low stringency binding to 3′ regions of unrelated messages [16] can lead to posttranscriptional repression or, respectively, the blockage of translation of other target sequences.

In view of the central role of the transcription factor STAT6 in asthma, the efficacy of different STAT6 siRNA-oligonucleotides to downregulate the STAT6 protein was investigated. Effective STAT6 siRNAs were able to block IL-4 dependent

*Corresponding author. Fax: +41 7351 545155.
E-mail address: silke.hobbie@bc.boehringer-ingelheim.com (S. Hobbie).

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production of eotaxin-3 in a human bronchial epithelial cell line. Furthermore, no unspecific effects of STAT6 siRNAs on STAT6 independent pathways were seen. Taken together, these data suggest that STAT6 siRNAs might have a therapeutic potential.

2. Materials and methods

2.1. siRNA preparation

Single stranded RNA-oligonucleotides were synthesized and 2′-OH deprotected at Dharmacon, Inc. (Lafayette, USA). Annealing of complementary oligonucleotides according to the manufacturer's instructions was controlled on 4% NuSieve low melting agarose gels. The design of the siRNAs followed mainly the rules of Tuschl [17]. The number coding of the siRNAs resembles the position of the siRNA sequence within the EMBL U16031.1 STAT6 sequence version. The sense sequences of the siRNAs were 5′-CAGUUCCGCCACUUGCCAAdTdT-3′ (si502), 5′-AGCCUGGUGGACAUUUAUUdTdT-3′ (si853), 5′-GAUGUGUGAAACUCUGAACdTdT-3′ (si1515), 5′-CAGAUGGGUAAGGAUGGCAdTdT-3′ (si2062). Control experiments were performed with siRNA specific for *lac*Z. After 5′-FITC labeling of the sense strand (5′-FITCUGGCGAUUACCGUUGAUGUdTdT-3′), this control was used in addition for the evaluation of transfection efficiencies.

2.2. Cell culture, siRNA transfection and cytokine stimulation

Human cell lines were exponentially grown under standard conditions. The growth media were RPMI 1640 (Gibco) supplemented with Glutamax (Invitrogen) for Jurkat, DMEM (Invitrogen) for HEK293 and HamsF12 (Cambrex) for BEAS-2B cells. Media were supplemented with 10% FCS (Hyclone). For transfection experiments, 4×10^5 cells per well were seeded in a 6-well plate and incubated overnight in normal growth medium to reach 50–70% confluency. Cells were transfected with 100 nM siRNA in the presence of 10 μl Lipofectamine 2000 (Invitrogen) in 2 ml of normal growth medium. Transfection efficiency was controlled 4 h after the addition of FITC labeled siRNA under a fluorescence microscope. 32 h post transfection, the cells were stimulated for 16 h by addition of fresh growth medium, supplemented with human TNFα (100 ng/ml), human IL-4 (50 ng/ml) or human EGF (50 ng/ml). The conditioned medium was stored at −20 °C for further analysis of the cytokine content and the cells were harvested by the addition of lysis reagents.

2.3. RNA isolation and qRT-PCR

Cells were lysed by the addition of 700 μl per well of RLT buffer and the total RNA was isolated from the lysate according to the RNeasy protocol (Qiagen). Briefly, genomic DNA was sheared by centrifugation of the lysate through qiashredder™ spin columns ($10\,000 \times g$, 2 min, Qiagen) and the RNA was precipitated by the addition of 1 vol. of 70% ethanol. The precipitate was applied on RNeasy spin column (Qiagen), DNAse treated and eluted in 60 μl DEPC-H_2O according to the manufacturer's protocol.

The gene expression levels were determined by TaqMan analysis in a 7900HT Sequence Detection System (Applied Biosystems) using TaqMan EZ RT-PCR reagent kit (Applied Biosystems) for the reverse transcription and PCR amplification in ABI PRISM 384 well optical reaction plate (Applied Biosystems). Gene specific probe was labeled with 6-FAM™ and TAMRA for internal quenching. Primers- and Mn^{+2}-concentrations were optimized to reach 100% amplification efficiency according to the manufacturer's instructions. For the normalization of TaqMan®, GAPDH control reagents were used (Human) (JOE™ Probe, Applied Biosystems). The gene specific forward-, reverse- and probe primer sequences were 5′-CCTTTTGGCAGTGGTTTGATG-3′, 5′-GTTTGCTGATGAAGCCAATGATC-3′, 5′-CCTCACCAAACGCTGTCTCCGGA-3′ for STAT6 and 5′-GTGCCAGCCTGGTTTGGT-3′, 5′-AGCTGGCTGACGTTGGAGAT-3′, 5′-AGACGACCTCTCTGCCCGTTGTGG-3′ for STAT1. The SOCS1 gene expression was analyzed by the human SOCS1 specific TaqMan® gene expression assay (Hs00705164_s1, Applied Biosystems).

2.4. Western blot analysis

Cell lysates (5×10^5 cells/lane) were separated by SDS–PAGE and transferred to Immobilon-P Transfer Membrane (Milipore, Bedford, MA, USA). Blots were blocked in 1× TBS/5% milk-powder overnight and incubated with anti-STAT6 (sc-621; 10 μg/ml; Santa Cruz Biotechnology, Santa Cruz, USA) or anti-STAT1 (sc-346; 10 g/ml; Santa Cruz Biotechnology, Santa Cruz, USA), washed three times with 1× TBS/0.1% Tween20 and incubated with HRP-conjugated secondary antibody. For the analysis of different protein levels on the same blot, membranes were stripped with ReBlot™ Western blot recycling kit (Chemicon). Immunoreactive bands were visualized using ECL (Amersham Biosciences Europe GmbH, Roosendaal, Netherlands).

2.5. Cytokine ELISAs

Concentrations of eotaxin-3 (CCL26) and IL-8 (CXCL8) in the cell supernatant were determined with human eotaxin-3 Quantikine (R&D systems, DCC260) and with OptEIA human IL-8 Set (BD/Pharmingen, # 555244).

2.6. Electrophoretic mobility shift assay

BEAS-2B cells were pelleted by centrifugation at $1000 \times g$, washed once in PBS and lysed in lysis buffer (20 mM HEPES pH 7.4, 100 mM NaCl, 50 mM NaF, 1 mM DTT, and 10% glycerol) by freeze–thawing. After three freeze and thaw cycles (RT, liquid N_2), the lysate was centrifuged at $10\,000 \times g$ for 5 min at 4 °C and the supernatant was used after the quantification of protein concentration for the EMSA. 5 μg protein was reconstituted in Band Shift Buffer (10 mM Tris HCl, pH 7.5, 50 mM NaCl, 1 mM DTT, 1 mM EDTA, 0.5 mg/ml BSA, 0.2 μg/ml pdIdC, and 5% glycerol) and incubated with 50 fmol DNA probe end-labeled with [α-^{32}P] ATP for 15 min at 4 °C. STAT6 probe: 5′-AGATTAAAGTTCCTGTGAAGGTCTT-3′ (STAT6 binding sequence out of the IL-4 promoter), STAT1 probe: 5′-AGCTTCATTTCCCGTAAATCCCTA-3′. Samples were separated by electrophoresis on 4% polyacrylamide gels in 0.5% TBE (Tris-borate EDTA) and bands were visualized by autoradiography.

3. Results

3.1. Identification of STAT6 specific siRNA

The knock down efficiencies of the STAT6 siRNA molecules were first analyzed in HEK293 cells due to the high transfection efficiencies achievable in this cell line. The knock down efficiencies after transient transfection of the siRNAs were analyzed by STAT6 specific qRT-PCR. Transfections without siRNA or with the *lac*Z specific siRNA served as a control for transfection reagent and double stranded RNA induced general silencing of transcription. Transfection efficiency was analyzed 4 h after transfection by fluorescence imaging and more than 90% of the cells were stained positive by an intracellular speckled pattern of the FITC-labeled oligonucleotides. As shown in Fig. 1, the remaining STAT6 mRNA levels 48 h after the transfection were 16 ± 4% for si502, 35 ± 16% for si853, 26 ± 10% for si1515 and 36 ± 17% for si2062, compared to control [no siRNA, control].

3.2. Knock down of STAT6 in BEAS-2B cells and blockage of IL-4 signaling

As a next step, the functional effects of a STAT6 knock down were analyzed in the human bronchial epithelial cell line BEAS-2B in which the STAT6 pathway has been described in detail [18]. Cells transfected with the two most effective siRNAs, si502 and si1515, or with the *lac*Z specific siRNA as control were stimulated with TNFα and IL-4 in order to increase the STAT6 gene expression and to activate the STAT6 signaling pathway. The effects of the treatments were first analyzed on the mRNA level by qRT-PCR (Fig. 2). The cytokine stim-

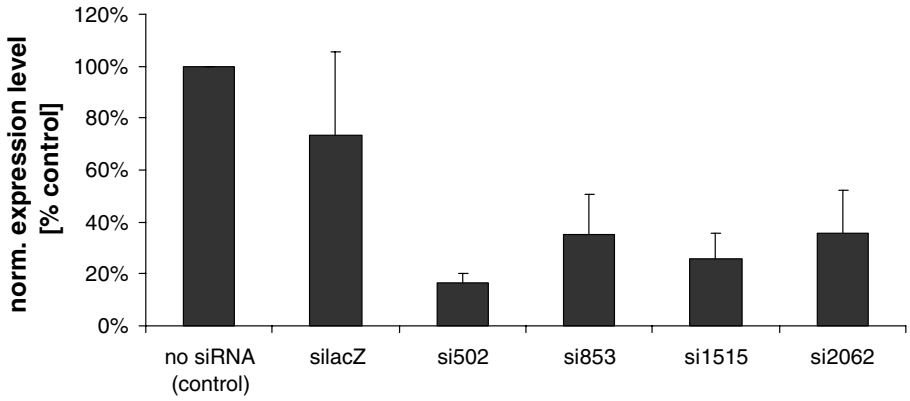

Fig. 1. Identification of potent STAT6 siRNAs: HEK293 cells were transfected with siRNAs specific for STAT6, *lac*Z or mock transfected. After 48 h, the cells were lysed and the knock down efficiencies of the siRNAs were analyzed by qRT-PCR and normalized for the GAPDH expression levels. The STAT6 expression level of the mock transfected cells was set as 100%. Results represent the mean of triplicate experiments including standard deviations (S.D.).

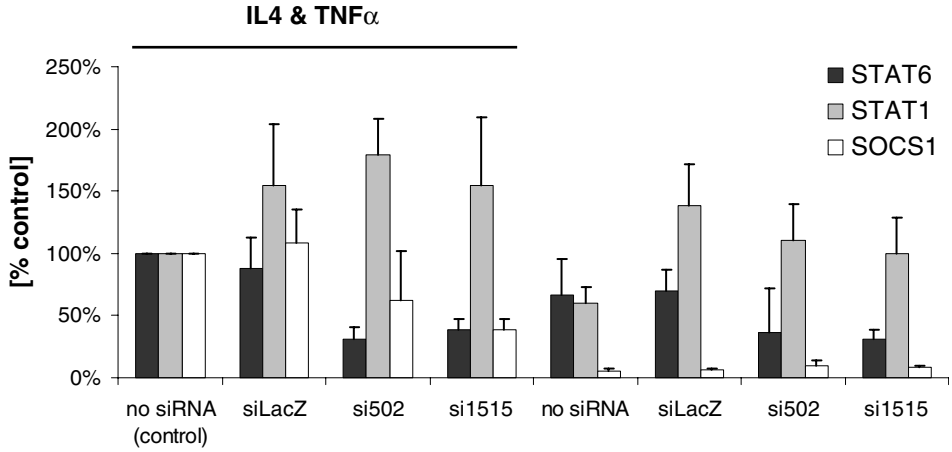

Fig. 2. Gene expression of STAT6, STAT1 and the target gene SOCS1: SOCS1 gene expression follows the reduction of STAT6 expression level and is not induced by the upregulation of STAT1. BEAS-2B cells were transfected with siRNAs and stimulated after 32 h with IL-4 and TNFα for 16 h. STAT6, STAT1 and SOCS1 mRNA levels were determined by qRT-PCR from cell extracts. Mock transfected cells were set as 100% and results represent means of six (except unstimulated siLacZ $n = 4$) experiments, including standard deviations (S.D.).

ulation of the cells resulted in a 1.5-fold increase from 67% [no siRNA] to 100% [no siRNA, control] in the level of STAT6 mRNA. Treatment of the cells with the *lac*Z specific siRNA had marginal influence on this cytokine stimulated regulation. In the presence of STAT6 specific siRNA molecules, si502 and si1515, the mRNA levels of STAT6 were reduced in the cytokine stimulated cells to 31% [si502] or 39% [si1515] of the control cells [no siRNA, control]. In unstimulated cells, the STAT6 mRNA levels were also reduced to 36% [si502] and 31% [si1515] compared to the same control of cytokine stimulated cells [no siRNA, control].

The STAT1 gene expression in these samples showed a consistent upregulation upon siRNA treatment regardless of the siRNA sequence. The maximum fold changes were 2.3-fold for siLacZ in the unstimulated cells (60% [no siRNA] versus 139% [siLacZ]) and 1.8-fold for si502 in the cytokine stimulated cells (100% [no siRNA, control] versus 179% [si502]).

The SOCS1 gene expression was used as a marker for JAK/STAT signaling. The regulation of SOCS1 gene expression followed the STAT6 expression levels: only background expression levels were seen in all unstimulated cells. Upon Il-4/TNFα stimulation, the expression level increased 10-fold in control and siLacZ treated cells. Knock down of STAT6 in cytokine stimulated cells resulted in 62% [si502] or 39% [si1515] of SOCS1 gene expression compared to control treated cells [no siRNA, control].

Western blot analysis of total cell lysates (Fig. 3) demonstrates a strong reduction of STAT6 protein in the siRNA treated samples compared to the control samples regardless of the absence or presence of the cytokine stimulation. This protein knock down was more pronounced for the si502 compared to the si11515 siRNA. Specificity of the RNAi activity on protein levels was assessed by the STAT1 specific Western blot, which showed unchanged protein levels regardless of cytokine stimulation or siRNA treatment.

To address whether the reduced STAT6 protein levels would result in the silencing of the STAT6 signal cascade, an in vitro DNA binding assay was performed (Fig. 4). Specific binding of STAT6 to the IL-4 promoter region was observed after cytokine stimulation (lanes 1 and 3) and was undetectable in the siRNA treated samples (lanes 5 and 7). As a control for specificity, the signals could be super-shifted by incubation of the

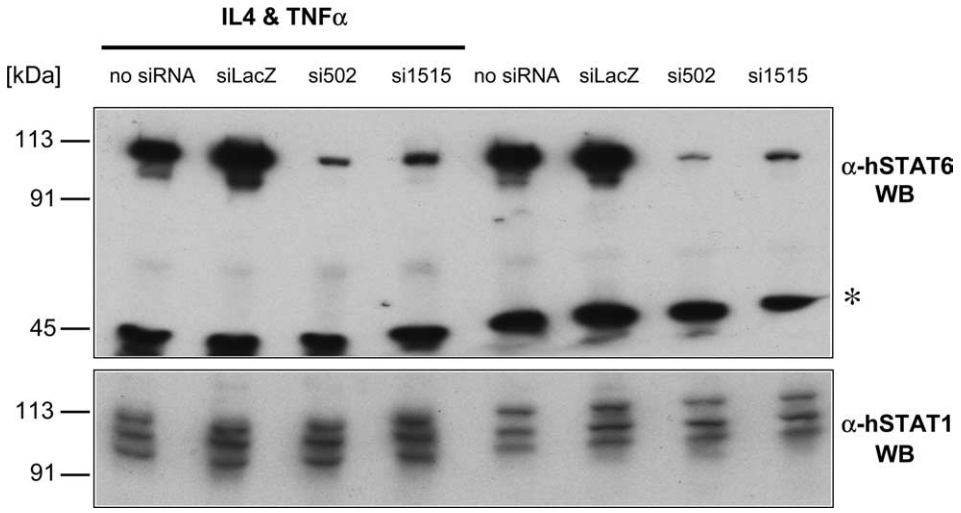

Fig. 3. Reduction of STAT6 protein by STAT6 specific siRNAs: BEAS-2B cells were transfected with siRNAs specific for STAT6, *lac*Z or mock transfected. After 32 h, the cells were stimulated with IL-4 and TNF for 16 h prior to cell lysis. Western blot was analyzed by specific antibodies for STAT6 or, after stripping of the STAT6 signal, for STAT1 as indicated. STAT6 Western blot signals of unknown protein served as loading control (marked with asterisk).

samples with a STAT6 specific antibody. Furthermore, no activation of STAT1 was observed using a STAT1-specific DNA probe (Fig. 4).

3.3. Specificity control of STAT6 knock down on the EGF signaling pathway

In a final set of experiments, it was addressed whether the siRNAs would specifically interfere with STAT6 mediated signal transduction or impact unrelated signaling pathways. In BEAS-2B cells, EGF leads to AP-1 and NF-κB dependent secretion of IL-8. As a cellular readout, the eotaxin-3 release from BEAS-2B cells stimulated with IL-4 and TNFα and the costimulation with EGF for the release of IL-8 was chosen. As shown in Fig. 5, the eotaxin-3 concentrations in the culture supernatant were at background levels (3%) in unstimulated cells and increased about 33-fold after stimulation of the cells with IL-4 and TNFα. Treatment of the cells with STAT6 specific siRNAs prior to the cytokine stimulation resulted in a tenfold decreased response of eotaxin-3 secretion (13% [si502] and 10% [si1515] compared to 100% [no siRNA, control]). As shown by the IL-8 secretion, BEAS-2B cells were fully competent for the AP-1 and NF-κB dependent signaling. Treatment of the cells with EGF resulted in a tenfold increased secretion of IL-8 into the supernatant regardless of the siRNA treatment and the pronounced reduction of the eotaxin-3 secretion.

4. Discussion

The data presented demonstrate that we identified two selective STAT6 siRNA molecules which were able to reduce STAT6 mRNA and protein levels in different cell lines. The depletion of STAT6 resulted in a loss of IL-4 and TNFα mediated stimulation of the eotaxin-3 release. Although we found

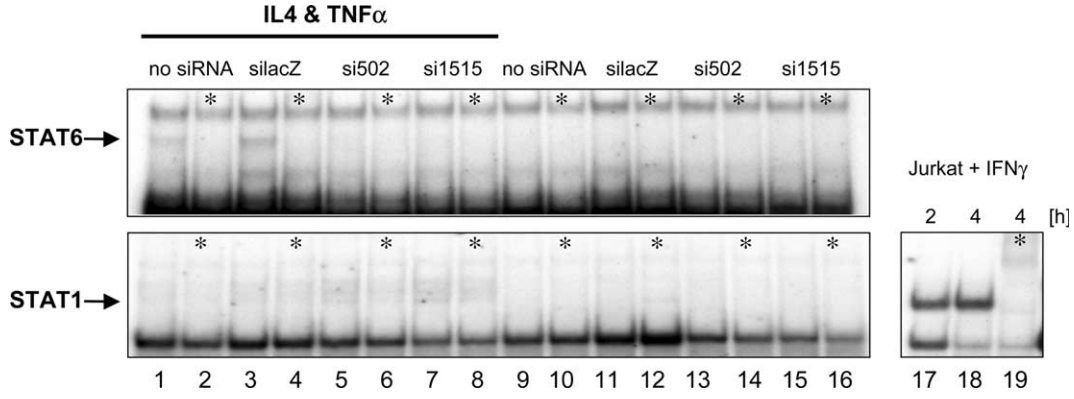

Fig. 4. Reduction of STAT6 expression by specific siRNAs abrogates the DNA binding of activated STAT6: BEAS-2B cells were stimulated for 16 h with IL-4 and TNFα after transient siRNA transfection. Cell lysates were incubated with labeled STAT6 or STAT1 specific DNA probes and separated by 4% PAGE. Binding of activated STAT6 is leading to reduced electrophoretic mobility as indicated by the arrow (lanes 1 and 3). As positive control for STAT1 DNA binding activity, the band shift of STAT1 specific DNA probes incubated with lysates of IFNγ (100 U/ml) stimulated Jurkat cells is shown (lanes 17 and 18). As a control for specificity, binding of the probe to STAT6 or STAT1 was blocked in the presence of specific antibodies (lanes marked with an asterisk).

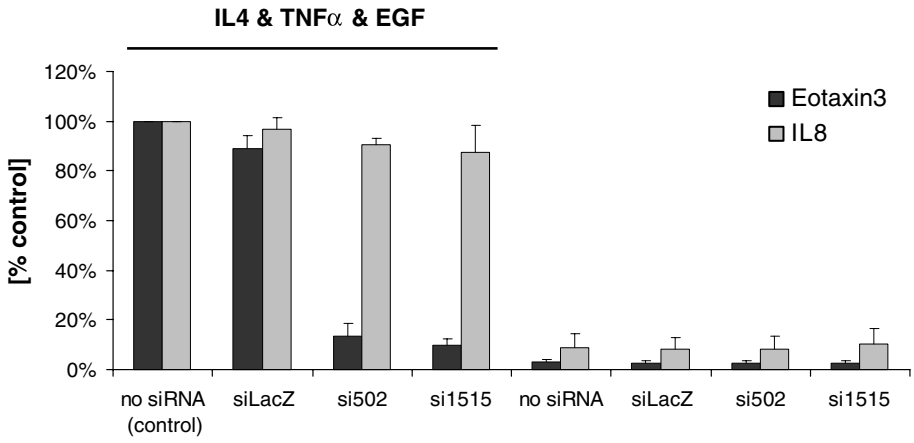

Fig. 5. EGF-mediated activation of IL-8 secretion is not blocked by STAT6 specific siRNAs: BEAS-2B cells were transfected as described and stimulated with IL-4, TNFα and EGF for 16 h. The supernatants were analyzed by ELISAs for the content of eotaxin-3 and IL-8. Results represent means of triplicate experiments, including standard deviations (S.D.).

an increased expression of STAT1 probably due to the high concentration of the siRNAs used in our assays, the expression had no impact on protein levels or DNA binding activity of STAT1. The negative crosstalk of the STAT6 and the STAT1 pathways was further excluded by the analysis of the SOCS1 gene expression. Upregulation of SOCS1 was only found in IL-4/TNFα stimulated cells without indication of an IFNγ mediated regulation in the presence of siRNAs. Therefore, we conclude that the STAT6 silencing was highly specific leaving related pathways untouched. The value of these siRNA molecules can be estimated from the high demand of alternatives to the corticosteroids actually used as standard asthma therapeutics and the lack of small molecule therapeutics able to interfere with this inflammation pathway. The further use of the RNAi technology will be highly dependent on the application route for in vivo experiments and therapeutic treatment. In this study, we complexed the RNA-oligonucleotides to enable the uptake into adherent cell cultures. Despite the fact that TNFα specific siRNAs complexed with DOTAP were used to prevent LPS induced sepsis in mouse peritoneum [19], the therapeutic use of this method is limited. Recently, the application of uncomplexed oligonucleotides became feasible by the injection of large volumes into the tail vein of mice [20,21] mainly affecting the expression of liver genes. Moreover, Zhang et al. [22] used the intranasal administration to deliver siRNAs to the mouse airway epithelium and parenchyma. These data suggest that a direct application of siRNAs into the lung might be feasible.

The STAT6 siRNAs described here may offer a novel approach to block STAT6 pathways in the airway epithelium after delivery to the lung. The blockage of STAT6 should lead to a reduced mucus production accompanied by a decreased eosinophilia and low 15-lipoxygenase activity [23,24]. Effects on underlying cell layers are expected for the remodeling of extracellular matrix (ECM) and airway hyper-responsiveness due to a decreased IL-13 signaling [25]. Reduced STAT6 expression in the blood system should interfere with Th2 differentiation, IgE switch, B-cell proliferation and mast cell activity [26–29]. Up to now, these effects are only speculative but will be addressed in further animal experiments.

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